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(54) Title: TRUNCATED IGF-I

(57) Abstract

The invention relates to a truncated variant of IGF-I having 1-69 of the amino acids of authentic IGF-I (IGF-I(1-69)) and a method of obtaining the truncated IGF-I either by expressing of IGF-I in yeast cells, enzymatic cleavage and thereafter isolating the truncated IGF-I from the medium or by introducing a plasmid encoding for the truncated variant of IGF-I and expressing the truncated variant of IGF-I. The invention also relates to a pharmaceutical composition containing this new truncated variant of IGF-I and to the use of IGF-I(1-69) for the preparation of a medicament.

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TRUNCATED IGF-I

The invention relates to a truncated variant of IGF-I having 1 - 69 of the amino acids of authentic IGF-I (IGF-I (1-69)) and a method of obtaining the truncated IGF-I either by expressing of IGF-I in yeast or bacterial cells, enzymatic cleavage and thereafter isolating the truncated IGF-I from the medium or by introducing a plasmid encoding for the truncated variant of IGF-I and expressing the truncated variant of IGF-I.

The invention also relates to a pharmaceutical composition containing this new truncated variant of IGF-I and to the use of IGF-I(1-69)for the preparation of a medicament

Insulin-like growth factor- I (IGF-I) is a growth factor comprising 70 amino acid as a single polypeptide chain, which displays relatively high homology with proinsulin. It is known to partly mediate the effect of growth hormone and also to display insulin-like properties. The human variant is nonglycosylated.

Human IGF-I with 70 amino acids is here called authentic IGF-I or rhIGF-I. The claimed truncated variant is IGF-I (1-69) or rhIGF-I (1-69).

Recombinant human insulin-like growth factor I (rhIGF-I) is produced by expression either in the yeast S. cerevisiae and is transported into the fermentation medium using the alpha-mating factor secretion system or in a bacterial cell.

Expression of foreign proteins in yeast can lead to post-translational modifications. One such modification that has been observed in proteins produced in yeast is glycosylation.

Yeast is known to contain carboxypeptidases (JONES, E. W., (1990), "Tackling the protease problem in Saccharomyces cerevisiæ", Methods Enzymol., 194, 428-453). Hence, the expression of foreign proteins in yeast may lead to production of C-terminally truncated variants.

Truncated variants of IGF-I are earlier known from WO 9118621 in which des (1-3-) - IGF-I is disclosed, useful in treating diabetes. Also the authentic IGF-I in which the glutamic acid at position 3 is replaced by another amino acid or deleted is known, see also WO 9110348. WO 9308826 discloses (4-70) IGF -I and (54-67)IGF-II, the truncated variant for promoting the survival of retinal neuronal cells in ophthalmic compositions. The truncated variant des(1-3) IGF-I is also known for reducing the severity of CNS damage. (WO 9302695).

WO 8701038 discloses peptide analogues of IGF-I in which 1-5 amino acid residues are absent from the N-terminal. They are said to have increased biological potency, useful in e.g. treating growth deficiency and catabolic disorders.

Here we describe the a new variant of C-terminally truncated IGF-I lacking the Ala70 residue of the authentic IGF-I. The polypeptide comprising 1-69 of the amino acids constituting IGF-I which is non-glycosylated, has normal, growth promoting IGF-I in vitro and in vivo, and exhibits the same IGF-I receptor binding as authentic human IGF-I.

It has a decreased lipogenic activity relative to IGF-I in vitro, and decreased insulin- like activity. This has been confirmed in vivo, demonstrating a less hypoglycemic activity than authentic IGF-I and, further, a lower affinity to the insulin receptor compared to authentic IGF-I. It has the same IGF BP3 binding

thus indicating a half-life similar to authentic IGF-I. Binding to IGF BP1 is the same as authentic IGF-I.

This new truncated variant of authentic IGF-I appears to be a promising therapeutical agent.

It must be regarding as surprising that truncation of only one amino acid at the C-terminal gives an effect since that part of the sequence is not supposed to be involved in e.g. insulin receptor binding.

The following figures are illustrating the invention:

Fig 1. Schematic representation of the plasmid construction
Fig 2. T/C response in cultured femora
Fig 3. Lipogenesis in adipocytes.
Fig 4. Lipogenesis in adipocytes
Fig 5. Binding to IGF binding proteins (IGFBP-1)
Fig 6. Binding to IGF binding proteins (IGFBP-3)
Fig 7 Binding to the insulin receptor
Fig 8a,b Binding to the insulin receptor
Fig 9. Glucose transport in Soleus muscle
Fig 10a,b. Binding to the type I IGF-I receptor and displacement of ^{125}I-IGF-I(1-69) (a) or ^{125}I-IGF-I (b)
Fig 11. Growth effects (Tibia growth) in vivo in hypophysectomized rats after 10 days of treatment
Fig 12. Anabolic action (urea concentration in serum) after 10 days treatment of hypophysectomized rats
Fig 13. Glucose concentration in hypophysectomized rats after single iv administration

THE INVENTION

The claimed IGF-I (1-69) can be produced by any means, such as isolating the truncated form from a medium, cleaving authentic IGF-I or direct expression by recombinant method.

The invention relates to a truncated variant of IGF-I having 1 - 69 of the amino acids of authentic IGF-I, IGF-1 (1-69), to a method of obtaining (1-69)IGF-1 e.g. by expressing authentic IGF-I in yeast cells and subsequent enzymatic cleavage of Ala 70 and isolating the IGF-1 (1-69) or by introducing a plasmid encoding for the truncated variant of IGF-I, expressing the truncated variant of IGF-I and isolating the IGF-1 (1-69). It relates to a pharmaceutical composition containing IGF-I according to claim 1 and a pharmaceutically acceptable carrier, diluent or excipient and to the use of this IGF-I variant for the preparation of a medicament for treating growth deficiences. The new truncated IGF-I (1-69) variant can also be used for the preparation of a medicament in treatments where authentic IGF-I is known to be effective, e.g. in the treatment of patients in a catabolic state, of patients with osteoporosis, for the preparation of a medicament for regeneration of tissues, for example peripheral nerves, central nervous system, muscle, skin and bone, for treating heart disorders, for example heart ischaemia, cardiac myopathy and congestive heart disorders and in diabetic patients with increased GH secretion.

The truncated IGF-I according to the invention should be given in doses which are therapeutically effective, preferably ranging from 10 µg/kg to 1 mg/kg, more preferably between 20 and 500 µg/kg body weigt.

Example 1

Preparation of C-terminally truncated variant IGF-I (1-69), Method A

The IGF-I gene was expressed in Saccaromyces cerevisiae using an alpha-mating factor leader peptide-IGF-I expression plasmid, p539/12. The process is described in detail in WO 90/02198, page 6 to page 7 under Exemple.

MATERIAL AND METHODS

Material

Recombinant human IGF-I was produced by Pharmacia AB and material was obtained from the manufacturing process of IGF-I, after the reversed phase high performance step, S5.

Lyophilized carboxypeptidase Y, (CPY), was purchased from Boehringer Mannheim (batch 13974820-32).

S-Sepharose fast flow (FF,) and Sephadex G-25 medium was purchased from Pharmacia Biotech.

Production Method

Cleavage and purification steps.

70 mg of CPY lyophilizate, containing 14 mg of CPY, was dissolved in 35 ml of 0.01 mol/l sodium phosphate buffer, pH 6.1. The CPY solution was mixed with 0.45 l IGF-S5 pool containing approximately 1.8 grams of rhIGF-I and incubated for 120 minutes at 35°C. After incubation the cleavage solution was cooled on an ice-bath. The cleavage solution was then applied at a linear flow rate of 300 cm/h to a 2.6 x 18 cm column packed with 95 ml of S-Sepharose FF gel. The gel was washed with 0.95 l of 0.01 mol/l sodium phosphate buffer, pH 6.1. Bound material was eluted with a 0.01 mol/l sodium phosphate/0.55 mol/l sodium chloride buffer, pH 5.9. The collected material had a concentration of 3.6 g/l

according to absorbance measured at 276 and the volume was 0.4 L. The batch contained >98% rhIGF-I(1-69) according to CZE (Capillary Zone Electrophoresis) and had a purity >98% according to RP-HPLC. A buffer exchange step was performed by running three cycles on a 5 x 35 cm column packed with Sephadex G-25. Equilibration buffer was 2.5 mmol/l sodium phosphate/88 mmol/l mannitol, pH 6.0. The protein peaks were collected from the three runs and fractions with an absorbance higher than 1.5 AU at 276 nm were pooled.

Dispensation

The concentration of the eluted and pooled protein solution was 2.7 mg/ml. The protein solution was sterile filtered through a 0.22 µm hydrophobic polyvinylidene difluoride membrane filter (Millipore Corp., USA), and then 3.7 ml portions were dispensed into sterile glass injection vials, made of type I borosilicate glass. After dispensing, each vial contained IGF-I(1-69) 10 mg, monosodium phosphate monohydrate 1.18 mg, disodium phosphate dodecahydrate 0.20 mg, and mannitol 59 mg. Rubber stoppers of grey bromobutyl rubber, FM157 (Helvoet, Belgium), were placed in freeze-drying position on the vials. The filtration and dispensing was performed aseptically under Laminar Air Flow hoods in a Class A environment. A total of 67 vials were filled. The vials were then transferred to a freeze-drier for lyophilisation.

Lyophilisation

The filled vials were placed on sterile freeze-drier shelves at room temperature. The vials were then frozen to -40°C for 8 hours. Primary drying was performed at 0°C and 0.2 mbar and secondary drying at +10°C and 0.2 mbar. To assure sufficient dehydration, measurement of pressure increase in the closed chamber was performed after each drying step before further progress. After lyophilisation pure nitrogen gas was introduced and the vials were stoppered

inside the freeze-drying chamber. The lyophilised product was called material obtained by method A.

Tests

The lyophilised material was found to be readily soluble in distilled water. The batch was tested for sterility and was found sterile. The content of endotoxins in the batch was also assayed and was found to contain less than 0.25 EU per mg protein.

Material for characterization

All biochemical characterization was performed on the lyophilized product.

Analytical methods

SAP V8 mapping.

The digestion of the rhIGF-I(1-69) material by Staphylococcus Aureus V8 protease (SAP V8) was performed with the concentration of sodium phosphate buffer used for the pH adjustment. The concentration was increased five times from 50 to 250 mmol/l.

The digest was separated on a reversed phase column, Zorbax 300 SB-C8 (5 µm, 4.6 x 250 mm), using 0.1% TFA in Milli Q water and 0.1% TFA in 60% acetonitrile/Milli Q water as eluent A and eluent B respectively. The chromatographic system was a Hewlett Packard 1090 M.

The gradient slope was:

Time (mins)	% A	% B
1	96	4
10	85	15
33	71	29
53	62	38
57	50	50
60	10	90

Detector wavelength: 215 nm

Column temperature: 37°C

Flow: 1 ml/min

Electrospray mass spectrometry (ES-MS).

Electrospray mass spectra recorded in the positive ion-mode by flow injection analysis (FIA) on a VG AutoSpec mass spectrometer equipped with an electrospray interface controlled by the OPUS software. The carrier stream for flow injection consisted of 1% formic acid in water-methanol mixture (50/50) pumped at 10 µl/min. The instrument was calibrated with a sodium doped PEG mixture, and 15 µl of the sample dissolved in the FIA carrier solvent was injected for analysis. Mass spectra were collected under continuum mode and typically 10 scans were average in the mass range of 200 - 2000 at a resolution of 1000 and a cycle time of 12 seconds.

RESULTS AND DISCUSSION

The CPY derived rhIGF-I(1-69) material was subjected to capillary zone electrophoresis, SAP V8 mapping, and electrospray mass spectrometry to verify the purity and the identity of the material. The purity was >98. The

migration of the product, compared to the rhIGF-I in-house standard is in agreement with the migration of rhIGF-I(1-69). The identity of the obtained product as rhIGF-I(1-69) was confirmed. The molecular weight of the intact material, 7577.6 rhIGF-I(1-69) agreed very well with the theoretically expected, 7577.6 Da.

Example 2

Preparation of (1-69)IGF-I by expression, Method B

In order to obtain sufficient material for biological characterization of this new variant of IGF-I, a plasmid encoding rhIGF-I(1-69) was constructed and rhIGF-I(1-69) was produced by expression in the yeast *Saccharomyces cerevisiae*. After purification and biochemical characterization, the material was submitted for biological characterization.

MATERIALS

Medium for fermentation

The medium used for culture in shaker flasks contained Yeast Nitrogen Base (without amino acids) 6.7 g/l, $NaH_2PO_4 \cdot 2H_2O$ 16.4 g/l, hydrolyzed casein 5.0 g/l, and glucose 20 g/l adjusted to pH 6.0. The mineral salt medium for the fermentation contained $(NH_4)_2SO_4$ 19.2 g/l, $NaH_2PO_4 \cdot H_2O$ 5.5 g/l, KH_2PO_4 4.5 g/l, hydrolyzed casein 26.0 g/l, $MgCl_2 \cdot 6H_2O$ 3.0 g/l, and $CaCl_2 \cdot 2H_2O$ 0.27 g/l. After sterilization, vitamins and trace elements solution were added. The final concentration of vitamins were (mg/l): biotin 0.032, folic acid 0.016, Ca-pantotenate 2.6, myo-inositol 12.8, nicotinamide 2.6, pyridoxine-HCl 2.6, riboflavinephosphate 3.4, thiamine-HCl 2.6, PABA 1.4. Trace elements added were (mg/l): $FeCl_3 \cdot 6H_2O$ 16.5, $ZnSO_4 \cdot 7H_2O$ 7.8, $CoCl_2 \cdot 6H_2O$ 0.30,

$Na_2MoO_4 \cdot 2H_2O$ 1.5, $CuSO_4 \cdot 5H_2O$ 0.46, KI 0.64, H_3BO_4 3.0, $MnSO_4 \cdot H_2O$ 2.4. IGF-I synthesis was induced by copper ions (CUP1 promoter), thus additional $CuSO_4 \cdot 5H_2O$ 50 mg/l was added from the start. All chemicals used were approved according to Pharmacia raw material specifications.

Materials for biological/biochemical tests.

Animals

Balb/c (clone 31) 3T3 fibroblasts were purchased from American Type Tissue Culture Collection (ATCC). Fertilized White Leghorn eggs were obtained from Linköpings Poultry Farm, Linköping. Male Sprague Dawley rats were supplied by BK Universal breeding laboratory, Stockholm.

Chemicals

Alfa-modified Eagles medium (a-MEM), Dulbecco's modified Eagles Medium (DMEM) and Trypsin-EDTA solution was purchased from Gibco (Life Technologies Ltd. Paisley, Scotland). Fetal calf serum was from HyClone (HyClone Laboratories, Inc.,Utah USA). Plastic tissue culture ware were from Falcon. Nonlabelled chemicals such as amino acids were obtained from Kebo. Collagenase, Glutamine, Transferrin, Dexamethasone, Penicillin/Streptomycin, Platelet Derived Growth Factor BB (PDGF BB), SDS, crystal violet and Bovine serum albumin were all from Sigma (Sigma Chemical company, St. Louis, MO, USA). Epidermal Growth Factor (EGF) was from an internal Kabi Pharmacia batch, 05073. Toluene scintillator was from Packard (Packard Instrument, Groningen, The Netherlands) and radioactive ^{14}C-glucose was purchased from DuPont Medical Scandinavia AB, Kista, Sweden).

Peptides

In house standard rhIGF-I preparations used as standard.

The IGF-I binding proteins; rhIGFBP-1 and IGFBP-3 were purchased from BioScience Center and from Mediagnost respectively.

Synthetic IGF-I 59-69

IGF-I 59-69 was synthesized by Solid-Phase technique on a Beckman Model 990 Synthesizer. The peptide was cleaved from the solid support by treatment with liquid HF/ anisole/ dimethylsulfide at 0°C, and purified by gelchromatography (Fractogel TSK HW-40, Merck).

The final productshowed to be > 90 % pure according to FPLC analysis (Column pep-RPC HR 5/5, Pharmacia), and amino acid analysis showed correct amino acid composition. Masspectrometry (FAB ionization) confirmed the correct molecular mass: Found: (M+H) 1208.6, Calc.: MW 1207.6 (monoisotopic).

METHODS

Plasmid and strain

Plasmid pX (Fig 1B), encoding rhIGF-I was constructed from pCGY1444 (Fig 1A) - a cloning vector similar to pCGY1413. In pCGY1444 a multilinker containing several restriction sites replaces the sequence encoding prepro-a-mating-factor-EGF in pCGY1413. pX was constructed by PCR amplification of the nucleotide sequence coding for prepro-a-mating-factor-IGF-I using p539/12 as the template and JABI43 (5'-CTGAATTCAAGAATGAGATT-3'), introducing an *Eco*RI site, and

JABI44 (5' -GTGGGCCCTTGGCTGCAGGTCGACGGA- 3'), introducing an *Apa*I-site, as oligonucleotide primers.

The amplified fragment was cleaved with restriction enzymes *Eco*RI and *Apa*I (Boehringer Mannheim), purified using Centricon 30 filters (Amicon) and subsequently ligated to the vector fragment of pCGY1444, cleaved with the same enzymes. The ligation mixture was digested with *Kpn*I to remove any intact pCGY1444 and transformed by electroporation with Biorad Gene Pulser according to Dower *et al* (1988) to *E. coli* RRIDM15 (Rüther 1982). Plasmids from transformants were prepared using QIAGEN-tip 20 or 100 and characterized by restriction digestion followed by manual DNA sequencing on double-stranded plasmid template or fragments subcloned in M13 using T7 Sequencing Kit (Pharmacia Biotech Sweden).

The plasmid encoding rhIGF-I(1-69), pXD70 (Fig 1C), was constructed as follows. A PCR amplification was performed with pX as the template and two synthetic oligonucleotides, JABI43 (se above) and ELMY13 (5'- AGTAGGGCCCCTAACTCTTCGCCGGTTTCAGCG-3'). ELMY13 contains the DNA sequence complementary to the 3'-end of the IGF-I sequence but lacks the codon for Ala70. Immediately after the stop codon an *Apa*I-site is introduced. The amplified 486 bp fragment was cleaved with *Eco*RI and *Apa*I, yielding a 478 bp fragment that was purified with an ultrafree Probind filter unit and a 30,000 NMWL ultrafree-MC filter unit (Millipore). pX was also cleaved with *Eco*RI and *Apa*I, yielding two fragments; 7,897 and 498 bp, respectively, which were separated by gel electrophoresis using 0.8% agarose. The gel slice containing the 7,897 bp fragment was cut out and purified using a QIAEX gel extraction kit. The 7,897 bp fragment was ligated to the cleaved and purified 478 bp PCR amplified fragment. The ligation mix was transformed to $CaCl_2$-competent HB101 cells (GIBCO BRL) and grown on ampicillin-containing agar plates. Colony-forming transformed cells were grown in 5 ml LB with 100 mg/ml ampicillin and plasmid preparations were made using QIAGEN-tip 20. The plasmids were cleaved with *Eco*RI and *Apa*I, analysed by gel electrophoresis (2% agarose) and the size of the smaller fragment (478 bp)

was compared to pX, cleaved with the same enzymes. Two of the colonies were grown in
50 ml LB with 100 mg/ml ampicillin and plasmid purification was performed using QIAGEN-tip 100. The DNA sequence of these two plasmids were analysed using an ALF (automated laser fluorescent) electrophoresis unit (Pharmacia Biotech). The sequencing reactions were performed with Pharmacia Autoread Sequencing Kit using fluorescein dATP as internal label. In figure 2 the sequence of the coding region of the expression unit is shown. The sequence of the cloned 478 bp fragment was verified by readings through the *Eco*RI-site using primer ELMY13 and through the *Apa*I-site using primer JABI43. Both plasmids contained the correct sequence. One of them, pXD70, was chosen to produce rhIGF-I(1-69). pXD70 was transformed to *Saccharomyces cerevisiae* Vn2, a yeast strain selected for resistance to Vanadate along the lines described by Chisholm *et al* (1990), by electroporation on a Bio-Rad Gene Pulser apparatus. The preparation of electrocompetent yeast cells and the electroporation was made according to Becker and Guarente (1991). Transformed cells were spread on selective SA (sorbitol agar) plates and colonies formed restreaked on YNB/CAA (trp-) plates.

Fermentation

The laboratory fermentations were carried out in a 75 l Pilot fermenter. The fermentation was started by inoculating an agar plate with 0.1 ml of the transformed Vn2 strain. The plate was incubated three days and then the cells on the agar plate were resuspended and transferred to a flask containing fresh medium, which was incubated 17.5 hours at 30°C on a rotary shaker. This culture was then transferred to the fermenter. The volume of the inoculum was 5.3 % of the working volume of the fermenter (30 l). The fermentation was run for 48 hours as a fed-batch culture maintained at pH 5.7, by titration with 25 % NH_4OH, and at 30°C. The aeration rate was set to 1 vvm (volume air/volume liquid/min) and the dissolved oxygen was controlled at 40% of saturation by

the stirrer speed. Glucose was added as a 612 g/l solution according to a profile linearly interpolated with time. After completed fermentation the broth was harvested by filtration in a Prostak filtration unit.

The fermentation was performed as a standard IGF-I fermentation (see Method A) when using the YE 465 host/vector system. The yield of filtrate after fermentation was 22 l containing 3 mg/l of rhIGF-I (1-69). 20 l of filtrate was purified.

The expression level of secreted rhIGF-I (1-69) was analyzed by RP-HPLC

Purification

Purification of monomeric rhIGF-I(1-69)

20 litres of yeast media from fermentation batch containing rhIGF-I(1-69) was clarified by centrifugation. 6.6 g/l citric acid$_{(s)}$ was added to the sample. The sample was adjusted with concentrated HCl to pH 3.0 and diluted with distilled water to an ionic strength of <24 mS/cm. The solution was loaded onto a column packed with Sulfonyl-Sepharose fast flow (Pharmacia Biotech) equilibrated with 80mM tri-sodium citrate, 41 mM sodium phosphate, pH 3.0, and the bound proteins were eluted with 0.1 M sodium phosphate, 0.1 M sodium chloride, pH 6.0.

Fractions containing rhIGF-I(1-69) analysed by RP-HPLC were pooled and loaded onto a column packed with Sephadex G-50 fine (Pharmacia Biotech) equilibrated with 50 mM sodium acetate, 0.1 M sodium chloride, pH 5.0.

Fractions containing monomeric rhIGF-I(1-69) were pooled for further purification and characterization.

Hydrophobic interaction chromatography and preparative RP-HPLC.

To obtain separation between correctly folded rhIGF-I(1-69) from misfolded isomers the gel filtration pool and an equal amount of 1.0 M sodium sulfate solution was mixed and applied to a 1.0x11 cm column packed with Butyl-Sepharose fast flow, equilibrated with 0.5M sodium sulfate, 10 mM sodium phosphate, pH 6.0.

The column was was hed with starting buffer and the bound protein was eluted with a gradient from 0.5-0.0 M sodium sulfate. The absorbance peak was collected.

The sample was loaded onto a 250x4.6 mm Hi-Chrom KR 100-10C8 column (EkaNobel) and eluted with a gradient from 24.4-27.1% acetonitrile. Six fractions were collected and desalted on PD-10 columns. Fraction six contained 85.5% rhIGF-I(1-69) according to CZE and was subject for further investigations and analysis.

Total recovery after the purification was 27 % and the purity obtained was 85.5 %

Characterization

Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) was performed on an Applied Biosystems Inc. 270A capillary electrophoresis instrument using a coated fused silica column, CElect-P150, from Supelco. On-line detection was performed 65 cm from the injection site and the detection wavelength was 200 nm. The samples were diluted to 0.2 mg/ml by 6 % 1-propanol, injected by pressure (2s) and analysed with an applied voltage of 25 kV. The column temperature was 30°C and the running buffer was composed of 60 mM sodium phosphate pH 2.0, 6 % 1-propanol and 0.1 % hydroxypropylmethylcellulose (HPMC).

SAP-V8 mapping

The digestion of the rhIGF-I(1-69) material by *Staphylococcus Aureus* V8 protease (SAP-V8) wasperformed in which the concentration of the sodium phosphate buffer was used for the pH adjustment. The concentration was increased five times from 50 to 250 mM.

The digest was separated on a reversed phase column, Vydac 218TP54 (5 mm, 4.6x250 mm), using 0.1 % TFA in Milli Q water and 0.1 % TFA in 60 % acetonitrile/Milli Q water as eluent A and eluent B respectively.

The gradient slope was;

Time (mins)	% A	% B
1	95	5
5	95	5
15	89.5	10.5
50	79.6	20.4
55	70	30
65	40	60

Detector wavelength: 215 nm

Column temperature: 37°C

Flow: 1 ml/min

RESULT

Biochemical characterization

The CZE analysis demonstrated a purity of 86 % and the migration time of the rhIGF-I(1-69) material agreed well with the migration time of the peak just ahead of the main peak, i.e. the C-terminally truncated rhIGF-I(1-69) form. The

agreement in migration times between the rhIGF-I(1-69) material(from method B) and the C-terminally truncated rhIGF-I(1-69) form (from method A) was also demonstrated by analysis of the rhIGF-I standard mixed with a minor amount of the rhIGF-I(1-69) material.

The three SAP-V8 maps originating from digestion of the rhIGF-I(1-69) material, the rhIGF-I standard mixed with a small amount of a synthetic modified SAP 9 peptide
(SAP 9* rhIGF-I(59-69), PCB 01203) and the rhIGF-I agreed well with one exception. The C-terminal part of the rhIGF-I(1-69) material eluted at the position of the synthetic SAP 9*peptide. This result was in agreement with earlier observations, hence the identity of the rhIGF-I(1-69) material with the found "impurity" was proven.

Isolation and characterization of this material proved it to be a C-terminally truncated form of the IGF-I molecule, lacking the amino residue Ala 70.

BIOLOGICAL ACTIVITY

Introduction.

Nuclear magnetic resonance data indicate that the core of IGF-I is structurally very similar to insulin. Structural determinants for IGF-I binding to the Insulin and Type I receptor in this region overlap. In agreement with this, IGF-I in addition to binding its own receptor crossreacts with the Insulin receptor, although in most cases with a much lower affinity.

The structure-function relationship of the IGF-I receptor is also similar to the insulin receptor. Apart from the different binding affinities for their respective ligands, the insulin receptor and the IGF-I receptor seem to be identical

regarding at least the initial steps in signal transduction following ligand binding. This suggests that bioeffect differences between IGF-I and insulin is mainly due to different expression of their receptors and signalling pathways in different target cells. An important determinant for the target organ bioeffects of IGF-I or IGF-I variants is therefore their affinity for, and bioeffects ,mediated by binding to the two receptors.

The differences between IGF-I(1-69) and authentic IGF-I with respect to their insulin-like activity in vitro has been investigated to show if a difference in binding to the insulin receptor was the major cause. Furthermore, we wanted to compare the effects of rhIGF-I(1-69) and authentic rhIGF-I in cells or tissues, carrying both insulin and type I IGF receptors. One such tissue is muscle, an important target tissue for insulin as well as IGF-I. Since skeletal muscle is the primary tissue responsible for glucose utilization and glucose transport across the cell membrane is the rate limiting step in glucose utilization, an in vitro model where isolated skeletal rat muscle was used to measure the transport of a glucose analogue, 2-deoxy-glucose, was set up. In this model rhIGF-I(1-69) were compared to authentic rhIGF-I.

In muscle, IGF-I and insulin also exert anabolic effects such as the ability to increase protein synthesis and decrease protein degradation (Gulve and Dice 1989). Assays for these effects were established in differentiated L6 rat muscle cells and rhIGF-I(1-69) was compared to authentic rhIGF-I.

Example 3

Biological characterization of the material obtained according to Method B.

METHODS

Bone growth promoting effect in vivo:

Femur Bioassay was performed as described below.

Fertile white Leghorn eggs were incubated in an egg-hatcher (Andersson and Bonde AB) in a humified atmosphere at 38 ± 0.5°C for 8 days. After sterilization of the eggshell with 70 % ethanol, the shell was broken with sterilized scissors and the embryo transferred to a sterile Falcon Petri dish. The two femora were carefully dissected out, and the adhering connective tissue removed by gently rolling the femora on dry filter paper. Isolated femora were then individually transferred to wells containing 0.5 ml of culture medium in 24 well tissue culture plates. The plates were incubated over night at 37°C in a CO_2-incubator under a humidified atmosphere of 5 % CO_2 in air. Following the over night incubation the femora were transferred to 6 well tissue culture plates containing sterile-filtered fresh culture medium +/- additions of the rhIGF-I preparations. When tested, the peptides were diluted to final concentrations of 20 and 40 ng/ml respectively. One femur of contralateral pairs was incubated in serum-free medium supplemented with peptides as described above, the other served as a pair-matched control and was incubated in medium alone. Groups of 6 bone-pairs were used for each test dose.

Control and test bones were incubated for 3 days at 37°C under a humidified atmosphere of 5 % CO_2 in air. n = 9 from two experiments.

At the end of the incubation period the medium was removed and replaced with 1 ml acetone for 5 minutes.The femora were then left to dry in air. The dry weight of each femur was measured on a precision balance and the growth promoting effect of the peptide estimated as the ratio of the treated (T) femur to that of its pair-matched (C) control.(T/C ratio).

The statistical significance was evaluated by Student´s unpaired T-test as based on mean values for 4-6 bone-pairs per assay.

Insulin-like effect

Isolated adipocytes were prepared according to Rodbell (1964). Briefly, dissected epididymal fat pads from 150-180 g rats were digested with shaking at 37° C for 45 min with collagenase (2 mg/ml) in Alpha Modification of Earles Medium (a-MEM) supplemented with 200 µM adenosine and 50 mg/ml bovine serum albumin. After filtration through cheesecloth and 2 washes in medium, the adipocytes were suspended in medium for the lipogenesis assay (Small et al 1987).

Adipocytes were incubated for 2 hours at 37°C with 0.1 µCi radioactive glucose/mmol glucose present in the medium, and 25, 50 and 100 ng/ml of the peptide to be tested. The incubation was interrupted by adding 5 ml/tube of toluene scintillator solution, followed by vigorous mechanical shaking to break open the cells. The tubes were left over-night to permit the extraction of lipids into the toluene phase, and finally counted on a liquid scintillation counter. Data are presented as means of independent tests with quadruplicate samples, unless otherwise stated.The statistical significance of differences in lipogenic response was evaluated by Student´s unpaired T-test.

Binding to the binding protein

A surface plasmon resonance technique (BIAcore Pharmacia Biosensor), was used to compare the binding activity for rhIGF-I and rhIGF-I(1-69) to their binding proteins (IGFBPs), the low molecular weight form, rhIGFBP-1 and the high molecular weight form, rhIGFBP-3. The rhIGFBPs were covalently linked to the dextran layer of the sensor chip surface. The binding to the rhIGFBPs of different dilutions of rhIGF-I

(1-69) were compared with rhIGF-I in house standard dilutions (DsQ 93). (Jönsson et al, 1991).

RESULTS

Biological characterization

Bone growth promoting effects

Within its wide spectrum of anabolic and mitogenic effects, rhIGF-I exerts stimulatory effects on bone- and cartilage growth. In *in vitro* organ culture of chicken embryo femora rhIGF-I was found to stimulate femoral growth in a specific, time- and dose-dependent manner. Femora incubated in the presence of 100 ng/ml rhIGF-I typically produced a 2-fold growth stimulation over controls. A ten-fold higher insulin dose was needed to evoke this effect. The data indicated that the bone growth stimulatory effects of rhIGF-I in the chick femur model were primarily mediated by the chick IGF-I receptor, since an equivalent to the mammalian IGF II/M6P receptor has not been found in avian species. When tested under standard conditions, rhIGF-I(1-69) was equipotent to the rhIGF-I in-house standard (DsQ 93) in its ability to stimulate bone growth. The estimated potency was 123,6 % (95 % confidence interval 73.9-135.4) relative to in house standard, which did not reach statistical significance compared to authentic rhIGF-I (Fig 2).

Lipogenesis

It is generally assumed that rhIGF-1 exerts its effects through the insulin receptor in adipocytes (King et al 1980, Massague et al 1982). We have demonstrated that insulin and rhIGF-1 are almost equipotent in their ability to stimulate lipogenesis in primary rat adipocytes, and no additive effects were observed when submaximal concentrations of rhIGF-I and insulin were combined, which suggested that lipogenic effects of rhIGF-I was mediated via a single receptor, most likely of the insulin-type.

The rhIGF-I(1-69) form demonstrated a significantly reduced ability to stimulate lipogenesis in rat adipocytes compared to authentic rhIGF-I. This was shown in two independent tests. In the first test, additions of 10 and 100 ng/ml were used. No response was seen when the low dose 10 ng/ml was added, while the higher dose, 100 ng/ml, of the reference preparation stimulated lipogenesis more than two-fold. rhIGF-I(1-69) also stimulated lipogenesis, but with a significantly reduced potency compared to reference rhIGF-I. This stimulation was approximately 60 % of the authentic rhIGF-I effect. When repeated with doses of 25, 50 and 100 ng/ml, all doses tested showed significantly reduced potency compared to in house reference. In this test 25 ng/ml standard rhIGF-I significantly increased lipogenesis while the same dose of rhIGF-I(1-69) had no effect. For the other doses tested, 50 and 100 ng/ml, the response was 63 and 74 % , respectively (Fig 3 and 4). Taken together the tests suggested that rhIGF-I(1-69) had a reduced lipogenic effect which was approximately 60 % of the authentic rhIGF-I.

Binding to the binding proteins (IGFBP-1 and IGFBP-3)

The binding of the rhIGF-I(1-69) preparation to rhIGFBP-1 and rhIGFBP-3 was approximately 70 % and 88 % respectively of IGF-I standard (response = 100 %). The binding of IGF-I and rhIGF-I(1-69) to the immobilized IGFBP-1 and IGF-BP-3, respectively, in Real-Time Biospecific Interaction Assay (BIA) are shown in Fig 5 and 6. The response was measured in resonance units (RU). This difference in response was not significant, indicating that the deletion of amino acid 70 did not affect binding to the binding proteins tested.

CONCLUSIONS

When rhIGF-I(1-69) was tested in three separate assays, measuring an IGF-I receptor mediated effect, no difference compared to the authentic rhIGFI was seen. Apparently the deletion of one amino acid in the C-terminal did not affect

binding to the IGF-I receptor. The binding to the binding proteins (IGFBP-1 and IGFBP-3) did not differ from that of the rhIGF-I reference . However, when an insulin receptor mediated effect was tested, a significant decrease in the lipogenic response (about 60 %) compared to that of the rhIGF-I in house standard was shown, indicating that the deletion selectively affects binding to the insulin receptor.

Example 4

Biological characterization of the material obtained according to Method A.

MATERIALS

Chemicals

L6 rat muscle cells were obtained from The American Type Culture Collection (ATCC). Dulbeccos Modified Eagles Medium (DMEM), Foetal Calf Serum (FCS), Penicillin and Streptomycin (PEST) and Phosphate Buffered Saline (PBS) were obtained from Gibco (Life Technologies Ltd.,Paisley, Scotland). ^{14}C-leucine and ^{3}H-2-deoxy-glucose were from DuPont NEN (Du Medical Scandinavia, Stockholm) and Ultima Gold from Chemical Instruments. Bovine serum albumin (BSA) and 2-deoxy-glucose were from Sigma Chemiclal Company (St.Louis, MO, USA), all other chemicals from Kebo. Alfa IR-3 mouse monoclonal antibodies against the type I receptor and monoclonal aEGF mouse IgG were supplied from Oncogene science (Novakemi; Stockholm). Bio Rad protein assay kit were purchased from Bio Rad Laboratories (Munich, Germany).

Peptides

Recombinant human IGF-I(1-69) produced in yeast (rhIGF-I) was produced from full-length IGF-I by Carboxypeptidase Y cleavage and purified. Identity was proved by SAP-V8 mapping and electospray mass spectrometry . Purity

was 97.6 % according to Capillary Zone Electrophoresis (Sonesson et al 1995). The material was lyophilized and dissolved in sterile water. Bovine insulin was purchased from Sigma.

Animals

Male Sprague Dawley rats with a weight of average 100 g were from BK Universal (Stockholm). The rats were kept under temperature and humidity controlled conditions, in plastic cages, and subjected to a 12 h light/12 h dark cycle, with free access to water and standardized pellet feed (BK Universal)

METHODS

To study the insulin receptor binding a modified radioreceptor assay (RRA) assay was developed based on an established IGF-I binding essay using Human Placental Membranes as the source for insulin receptors and an antibody, aIR-3, to specifically block binding to IGF-I receptors.

Receptor preparation

As receptor preparation a crude membrane from human placenta (PLM) was used. This crude membrane preparation had a concentration of 5.83 mg membrane protein/ml measured by Bio-Rad protein assay kit. For binding experiments this crude PLM was diluted to a concentration of 1 mg/ml in 50 mM Tris-buffer, pH 7.4.

Solubilized membranes were prepared as follows: The crude membrane preparation was mixed with 1% Triton X-100, centrifuged at 200 000 x g and reconstituted in Tris-buffer. The protein concentration was determined and found to be 3.16 mg/ml, thus, a recovery of 54% was achieved. For binding

experiments the solubilized PLM was diluted 1:2 to a final concentration of 1.58 mg/ml.

Antibody titration

Preliminary tests were performed to titrate an effective concentration of the mouse monoclonal antibody aIR-3, needed to block IGF-I binding.

In the experiments, a two-step dilution series from undiluted antibodies (stock concentration of 0.1 mg/ml) to a dilution of 1:128 i.e. from 2 µg to 16 ng was tested out. In the subsequent experiments an amount of 2 µg antibody was chosen, since it was found to be most effective in blocking the IGF receptors. As control, a monoclonal aEGF mouseIgG was used.

Binding assays

IGF-I-binding studies were performed using both crude PLM and solubilized PLM as receptor preparations.

The method used was essentially the same as in IGF-1- i.e. overnight incubation of membranes, in the presence of unlabelled IGF-1 and ^{125}I-IGF-1 (approximately 10 000 cpm) at +4°C, except that the membranes were preincubated 1 hour with 20 µl of antibodies/tube to specifically block IGF-I receptors.

To determine the efficiency by which the aIR-3 monoclonal blocked type I receptor binding under these conditions, a high concentration (10 µg/ml) of "cold" IGF-1 was added to some of the tubes to get a total displacement of the tracer.

Following the over night incubation the tubes were centrifuged at 5000 x g 15 min., the supernatants were discarded, the pellets washed with ice-cold buffer, recentrifuged and finally counted on a gamma-counter.

When solubilized PLM was used, the experimental conditions were the same, except that the membranes were precipitated with a 25% solution of PEG-6000 in phosphate buffer before a single 30 min. centrifugation.

When insulin binding was studies the method used was the same as above except that ^{125}I-insulin (30 000 cpm) was used as tracer and displaced with unlabelled insulin, rhIGF-I or rhIGF-I(1-69), respectively, in the absence or presence of 1 µg aIR-3 (instead of 2 µg as in previous experiments, since 2 µg was only slightly more effective than 1µg) and solubilized PLM, (1.58 mg/ml).

Glucose transport

Glucose transport activity was measured by the use of the non-metabolized glucose analogue 2-deoxy -D-glucose using a method modified from Dohm et al (1988) as described below:

Rats were sacrifized and intact soleus muscles were rapidly dissected out and preincubated in plastic scintillation vials containing 3 ml Krebs Ringer buffer (KRB), pH 7.4, 5 mM pyruvat and 10 mg/ml BSA, for 30 minutes on a shaking water bath at 37°C. After the preincubation the muscles were transferred to vials containing KRB buffer with the addition of 0.1 µl/ml ^{3}H-2-deoxy-glucose, 10 mM 2-deoxy-glucose with or without peptides to be tested. The incubations were carried out at 37°C in a metabolic shaker. The medium was vigorously gassed with a mixture of 95 %O_2 : 5 % CO_2 for 5 minutes immediately before use. After incubation, the muscles were washed for two hours in cold PBS, to remove extracellular ^{3}H-deoxy-glucose. Then the muscles were lyophilized, weighed and dissolved in 1 ml 1 M NaOH at 40°C. 10 ml Ultima gold scintillation solution was added and the samples were counted in a Packard Tricarb scintillation counter. The radioactivity in samples were corrected for

quenching by use of internal standards. Results were expressed as dpm per mg of muscle dry weight.

Protein synthesis/degradation

The methods used for measuring protein turnover was slightly modified from Ballard et al (1986). Protein content was measured as described by Bradford (1976) using a BioRad protein assay kit.

Protein synthesis was measured as described below: Following preculturing of L6 cells to the differentiated myotube stage, the tissue culture medium in each well of a 6-well tissue culture dish was replaced with serumfree medium. After an over night incubation the cells were washed twice with leucine free DMEM medium, and incubated at 37°C for 1 hour in this medium. The measurement began by replacement of the medium with fresh medium containing 0.5 µCi/ml ^{14}C-leucine (ie total leucine content 2 nmol). After labelling for 4 h the medium was washed with medium containing leucine and left for 1 hour at 37°C in the same medium. Each monolayer was washed twice with PBS and twice with 4 % TCA. The washed cells were lysed in 0.5 ml of 0.5 M NaOH for the measurement of protein and radioactivity. All incubations, except for the over night incubation, were made with or without IGF-I.

Rates of incorporation were normalized for protein content in each well.

Rates of protein degradation were determined using identical procedures as for measurement of synthesis up to the final incubation with ^{14}C-leucine. At the end of this incubation the medium was removed,and cell-associated radioactivity was determined after dissolving the monolayers. Radioactivity was also determined in the medium samples after TCA precipitation. After 1 h at 37° in 4 % TCA, samples were spun in a Microfuge for 2 min and acid-soluble radioactivity was determined. The total radioactivity released into the medium plus that remaining in the cell fraction was taken to represent the initial

radioactivity. The percentage of protein degraded was calculated by taking the the radioactivity present in the medium and dividing this with the initial radioactivity.

RESULTS

Binding

In the first binding experiments we tested the IGF (type I) receptor antibodies and determined an effective dose for blocking the IGF-I receptorsThe aIR-3 antibody blocks the IGF-1-receptor in a concentration dependent way, and 2 µg of antibodies was only slightly more effective than 1 µg. However, only 60 % of the total IGF-I binding was inhibited. Addtion of "cold" IGF-1 was more effective and displaced 95 % of the labelled IGF-I. Since IGF-1 is known to cross react with the insulin receptor, it is likely that excess "cold" IGF-I displaced labelled IGF-I also bound to insulin receptors. The control antibodies aEGF do not influence IGF-1-binding.

Experiments using solubilized PLM resulted in almost the same binding as with crude PLM. However, the difference between aIR-3 blocking of the IGF-1-receptor and total displacement was not as large as with crude PLM. AlphaIR-3 blocked 70 % of total IGF-1 binding and "cold" IGF-I displaced labelled IGF-I up to 90 %. This indicated that the antibodies blocked the IGF-1-receptor more effectively when solubilized membranes were used. Thus, solubilized PLM with the addition of 1µg alpha-IR3 was used for the insulin receptor binding study.

We , furthermore, calculated the dissociation constant of the antibody in order to compare with the dissociation constant of the high affinity binding site for IGF-I. Using Scatchard plot we calculated a preliminary K_d = 0.5 nM for the antibody for the high affinity site of IGF-I K_d = 1.5 nM was calculated. The

affinities of the antibody and IGF-I for the receptor were, thus, of the same magnitude. This was in agreement with Jacobs et al 1986, who reported that alphaIR-3 bound to human placental membranes with a affinity similar to that of IGF-I.

To test if the antibody affected insulin binding a preliminary test was done using labelled insulin, displaced with unlabelled insulin, rhIGF-I and rhIGF-I(1-69) and in the presence or absence of alphaIR-3. The results of this test (fig 7) showed that the alphaIR-3 did not influence insulin binding, displacement with 250 ng/ml unlabelled insulin or displacement with 500 ng/ml IGF-I/IGF-I(1-69). Fig 8 shows the displacement curves obtained without (fig 8a) and with (fig 8b) the addition of a constant amount (1µg) alphaIR-3. The antibody did not interfere with the binding of insulin and did not affect displacement of labelled insulin from the insulin receptor.

Competition by unlabelled insulin for ^{125}I-insulin binding demonstrated that halfmaximal displacement occurred at approximately 20 ng/ml. rhIGF-I was approximately 200 fold less potent than insulin with 50 % displacement occurring at approximately 4 µg/ml. The variant, rhIGF-I(1-69), demonstrated a 1000 fold less potency compared to insulin, with 50 % displacement occurring at 20 µg/ml. Thus, it is concluded that the ability of rhIGF-I(1-69) to compete for ^{125}I-insulin binding was around 20 % of that of authentic rhIGF-I.

Glucose transport

In intact rat soleus muscle a concentration of insulin 50 ng/ml has been shown previously to stimulate glucose transport by 75 % . At a similar concentration rhIGF-I produced a slightly lower effect, ie glucose transport was increased by 25 %).

To determine if rhIGF-I and rhIGF-I(1-69) differed in their ability to stimulate muscle glucose transport in vitro, experiments were performed where doses of 10 and 50 ng/ml of the two peptides were tested. Insulin, at these test doses, was included as reference. A dose dependent increase in glucose transport was shown for all peptides. 50 ng/ml rhIGF-I and rhIGF-I(1-69) stimulated glucose transport rate by 39 % and 52 %, respectively, while insulin at the same dose increased transport by 84%. No statistical difference in potency between the two IGF-I variants was demonstrated (Fig 9), whereas both were slightly less potent than insulin.

Protein synthesis/degradation

When fully differentiated L6 cells were incubated in serumfree medium in the presence of 100 ng/ml rhIGF-I, a 35 % increase in protein synthesis and a 15 % decrease of degradation rate was seen. In two separate experiments, rhIGF-I(1-69) was compared to authentic rhIGF-I and protein synthesis/degradation rates were examined. No significant difference between the two IGF preparations could be demonstrated, although at post maximal doses a trend towards a somewhat lower response to rhIGF-I(1-69) was noted. (fig 10 shows results from 1 experiment).

Example 5:

In vivo activity of IGF-I(1-69) according to method A

Growth promotion:

In order to study the growth promoting action in vivo of the variant IGF-(1-699 hypophysectomized rats were treated for 10 days and growth variables tested.

Materials:

Hypophysectomized male rats were purchased from Möllegaard Breeding Lab, Denmark. They were allowed to acclimatize after arrival for at least 4 days. Authentic IGF-I and IGF-I(1-69) from method B) were used. They were dissolved in saline and filled in osmotic minipumps (Alzet model 2002) at two concentrations each, giving daily doses of 300 µg/rat and day (High dose, HD) and150 µg/rat and day (Low dose, LD), respectively.

The minipumps were inserted subcutaneously in the neck region of the rats during slight anaesthesia onthe first day of experiment.

Human GH was given to rats at a dose of 60µg/rat and day continuously and saline treated rats were used as controls.

Each rat received one intraperitoneal injection of Tetracyclin (10 mg/kg) on day one. Tetracyclin was used as a marker of skeletal growth.

Food consumption was measured during the experiment, and body weight of the rats was recorded before and after the experiment. Total nitrogen, serum-urea, hormone levels in serum, skeletal growth and blood glucose was measured after 10 days treatment.

RESULTS:

No effects on glucose was observed in the rats treated in any group.

Body weight gain was highest in the GH treated rats. No difference in stimulation of weight gain was seen in groups treated with authentic IGF-I or IGF-I(1-69), but both IGF-I stimulated weight gain significantly compared to control. Skeletal growth, measured in tibia, was most pronounced in the GH treated group, but significant effects versus control were seen in both IGF-I and IGF-I(1-69) treated groups, Fig 11. No significant difference between IGF-I and IGF-I(1-69) treated rats were seen. Further anabolic effects were seen on serum

urea, Fig 12. where GH as well as IGF-I and IGF-I(1-69) all exhibited clearcut decreased values compared to control, thus demonstrating anabolic effects.

Insulin-like effects:

In order to confirm the difference in insulin-like activity between IGF-I and IGF-I(1-69) the hypoglycemic action of the two peptides was studied in vivo in hypophysectomized rats.

Materials:

Hypophysectomized male rats were purchased from Möllegaard Breeding Lab, Denmark. They were allowed to acclimatize in the lab for at least 4 days after arrival.

The animals were anaesthetized and an incision was done on a tail vein.

Blood samples were taken for glucos determination (basal level). The animals were then allowed to wake up before the experiment took place.

Each rat received one intravenous injection of either authentic IGF-I (20 µg) or IGF-I(1-69)(20 µg) and blood samples were taken at 15 or 30 minutes intervals up to 180 minutes after injection. Blood glucose was determined.

At least five days after the first test each rat was tested again, but this time the rats received a dose of glucose (50mmoles/L; 200 µl/rat) immediately after the hormone injection. Blood samples were taken as before.

Insulin (1µg/rat) was used as positive control, and saline was given as control.

RESULTS:

A clearcut decrease in blood glucose was observed with both authentic IGF-I and IGF-I(1-69) in hypophysectomized rats with normal blood glucose, as expected. When glucose was given at the same time as the IGFs there was a significant difference in the decrease in blood glucose between the two products, ie the effect of IGF-I(1-69) was clearly less than that seen after

authentic IGF-I, Fig 13, thus demonstrating that the hypoglycemic effect of IGF-I(1-69) is lower than that of authentic IGF-I in vivo.

These in vivo results confirmed the results obtained in vitro,and showed that IGF-I(1-69) has full growth promoting activity but decreased insulin-like activity.

DISCUSSION

The difference, in the bioactivity between the two peptides was the finding of a significantly lower lipogenic effect in primary rat adipocytes with rhIGF-I(1-69). These cells carry insulin receptors but not type I IGF receptors,and thus the assumption was that rhIGF-I(1-69) had a reduced ability to bind to the insulin receptor.

The data in this report support this assumption. Using a modified human placenta membrane insulin RRA we demonstrated that rhIGF-I(1-69) bound to the insulin receptor with a reduction of ~20 % in potency compared to authentic IGF-I.

IGF-I type I receptors are expressed on most cells and in a Receptor Assay both peptides were equipotent in their ability to bind to the IGF type I receptor.

Fig 10 shows displacement curves obtained when labelled rhIGF-I(1-69) was displaced with unlabelled rhIGF-I(1-69) and rhIGF-I and when labelled rhIGF-I was displaced with unlabelled rhIGFI and rhIGF-I(1-69). In both cases half maximal displacement was seen at approximately 10 ng/ml, and no difference between the peptides in binding to the type I IGF receptor was noticed.

Adipocytes (Zapf et al 1981, Di Girolamo et al 1986, Bolinder et al 1987), adult liver (Caro et al 1985, Venkatesan et al 1990) and certain B-cell lines (Lowe et al 1991) express insulin receptors but not type I IGF receptors. Thus the IGF-I

action on these types of cells are expected to be mediated mainly via the insulin receptors.

In other tissues, both receptor types (insulin and IGF type I) are expressed in variable relative numbers. Skeletal muscle e.g. contains significant numbers of type I IGF receptors, as well as insulin receptors. Insulin receptors demonstrate high affinity binding to insulin and low affinity binding for IGF-I, while the IGF-I receptors bind IGF-I with higher affinity than they bind insulin. Thus, differences in receptor affinities is expected to direct IGF-I action to its own receptor in tissues with both types of receptors, (Dohm et al 1990, Verspohl et al 1988). The data presented in this report demonstrated that rhIGF-I(1-69) and rhIGF-I were equipotent with respect to muscle glucose transport in intact muscle in vitro and to protein turnover in differentiated L6 muscle cells, indicating that the effect was mediated via the type I IGF receptor and not by crossreaction on the insulin receptor.

These data confirms that IGF-I and IGF-I(1-69) are equipotent in stimulating cell proliferation and chick femur growth in vitro.

In conclusion, C-terminal deletion of one amino acid (Alanine70) of the IGF-I molecule, resulted in a truncated molecule, rhIGF-I(1-69), which seem to be more selective for the type I IGF receptor than authentic IGF-I. Thus, rhIGF-I(1-69) was equally potent with authentic IGF-1 with respect to bindning to the type I IGF-1 receptor, but demonstrated reduced binding to the insulin receptor. Furthermore, it has been confirmed that the growth promoting, anabolic active of IGF-1(1-69) in vivo is equal to authentic IGF-1, whereas the insulin like activity, such as the induction of hypoglycemia in vivo or lipogenesis in vitro is significant decreased.

The IGF-I (1-69) analog has higher selectivity for IGF-I receptors than for insulin receptors. This indicates that the analog can be selective for tissues

expressing the type I IGF receptor for such as bone and muscles, but not for tissues mainly expressing the insulin receptor, such as fat and liver.

IGF-1(1-69) does not induce hypoglycemia to the same extent as authentic IGF-1 and may be given in higher doses.

It must be regarded as surprising that this effect is obtained inspite of direct involvement of the known epitope for the insulin receptor binding.

Reference list:

Ballard F J et al. Biochem J, 1986; 33; 223-230

Becker DM et al, 1991, 194, 182-87.

Bolinder J et al, J Clin Endocrin Metab, 1987; 65; 732-737

Bradford M. Anal Biochem, 1976; 72; 248

Caro J F et al, J Clin Invest, 1988; 81; 976-981.

Chisholm V et al, methods in Enzymology, 1990, 185, 471-82

Di Girolamo M, FEBS Lett, 1986; 205; 15-19

Dohm G L et al. Diabetes, 1990; 39; 1028-1032.

Dower W J et al, Nucleic Acids Research, 1988, 16, 6127-45.

Gulve E A et al, Biochem J, 1989, 260, 377-87.

Jacobs S et al. Endocrinology, 1986, 118; 223-226.

Jönsson U et el, Bio Techniques, 1991, Vol 11,5.

King GL et al, J Clin Invest, 1980, 66, 130-140.

Lowe W L et al. CRC Press, Boca Raton Fl, 1991; 49-85.

Massague J et al, J Biol Chem, 1982, 257, 5038-45.

Rodbell M J Biol Chem, 1964, 239, No 2, 375-380

Rüther, Nucleic Acids Research, 1982; 10,5765-72

Small J etal, J Biol Chem, 1987, 262, No 23, 11071-79.

Venketesan N et al. Am J Physiol, 1990; 258; E329-337.

Verspohl E J et al. J Clin Endocrin metabol, 1988; 67/1;169-174

Zapf J et al. Eur J Biochem, 1981; 113; 605-609.

CLAIMS

1. Truncated variant of IGF-I having 1 - 69 of the amino acids of authentic IGF-I, IGF-1(1-69).

2. A method of obtaining an IGF-I variant according to claim 1 by expressing authentic IGF-I in yeast cells and subsequent enzymatic cleavage of Ala 70 and isolating the IGF-1(1-69).

3. A method of obtaining an IGF-I variant according to claim 1 by introducing a plasmid encoding for the truncated variant of IGF-I, expressing the truncated variant of IGF-I and isolating the IGF-1(1-69).

4. A pharmaceutical composition containing IGF-1(1-69) according to claim 1 and a pharmaceutically acceptable carrier, diluent or excipient.

5. A method of preparing a pharmaceutical composition according to claim 4 comprising mixing IGF-1(1-69) according to claim 1 with a pharmaceutically acceptable carrier, diluent or excipient.

6. Use of IGF-1 (1-69) according to claim 1 for the preparation of a medicament for treating growth disorders.

7. Use of IGF-1(1-69) according to claim 1 for the preparation of a medicament for treating patients in a catabolic state.

8. Use of IGF-1(1-69) according to claim 1 for the preparation of a medicament for treating of patients with osteoporosis.

9. Use of IGF-1(1-69) according to claim 1 for the preparation of a medicament for regeneration of tissues, for example peripheral nerves, central nervous system, muscle, skin and bone.

10. Use of IGF-1(1-69) according to claim 1 for the preparation of a medicament for treating heart disorders, for example heart ischaemia, cardiac myopathy and congestive heart disorders.

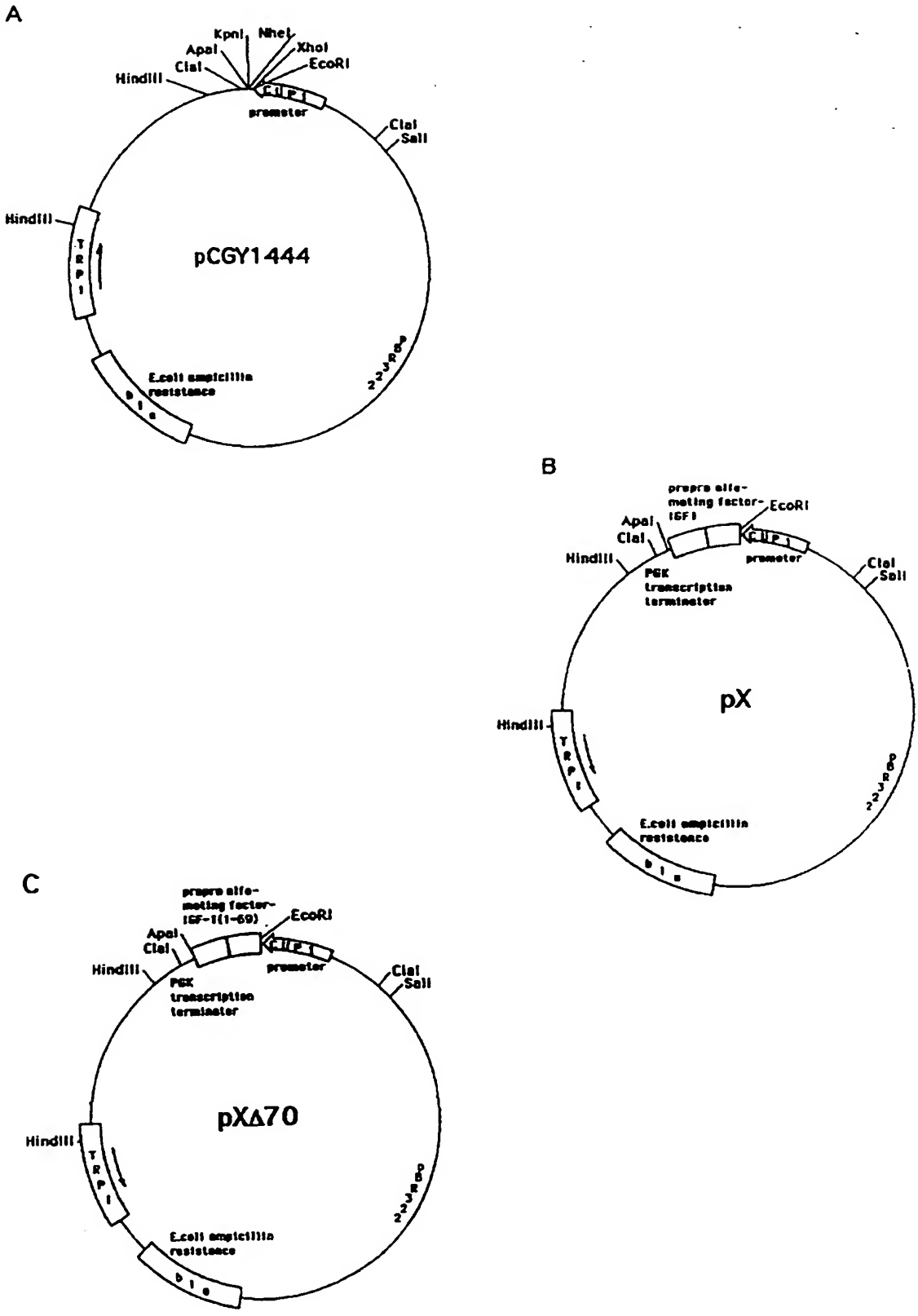

A
KpnI
NheI
ApaI
XhoI
ClaI
EcoRI
HindIII
promoter
ClaI
SalI
HindIII
pCGY1444
E.coli ampicillin resistance
B
prepro alfa-mating factor-IGF1
ApaI
ClaI
EcoRI
HindIII
promoter
ClaI
SalI
PGK transcription terminator
pX
HindIII
E.coli ampicillin resistance
C
prepro alfa-mating factor-IGF-1(1-69)
ApaI
ClaI
EcoRI
HindIII
promoter
ClaI
SalI
PGK transcription terminator
pXΔ70
HindIII
E.coli ampicillin resistance

Fig. 1

T/C response in cultured femora.
Comparison with the IGF-I in house standard.

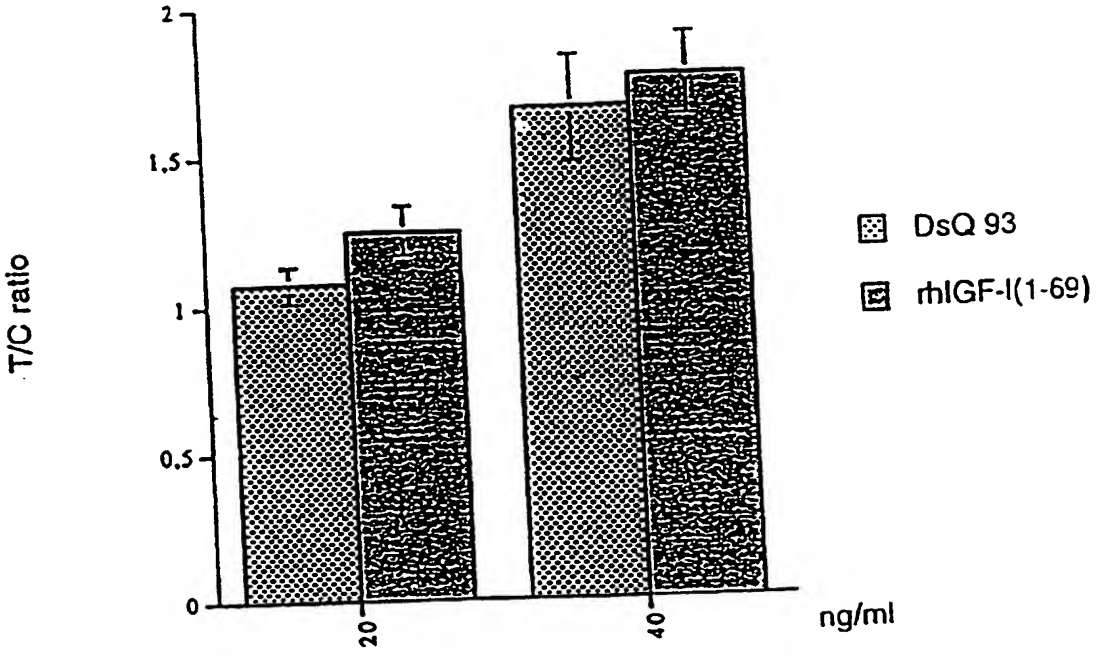
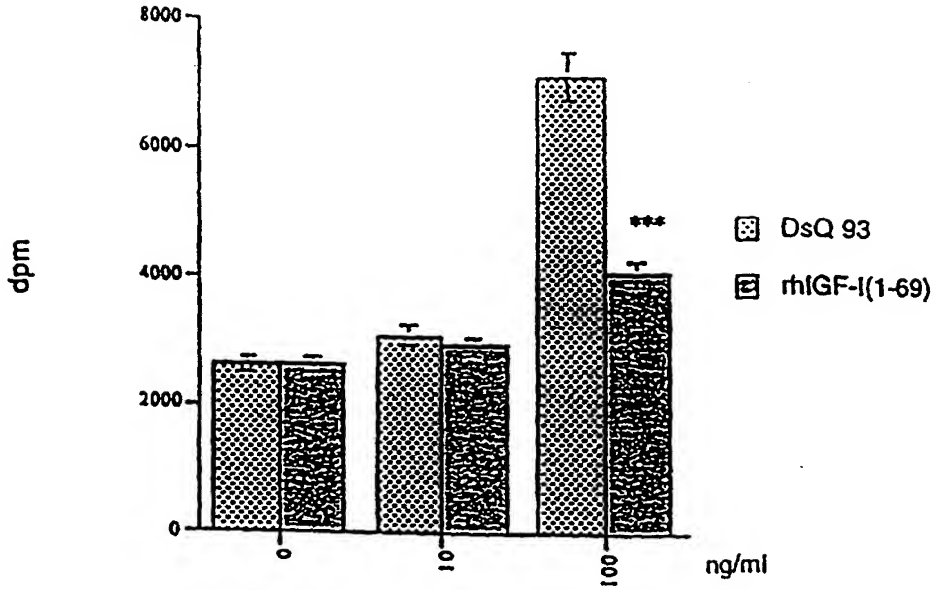


Fig. 2

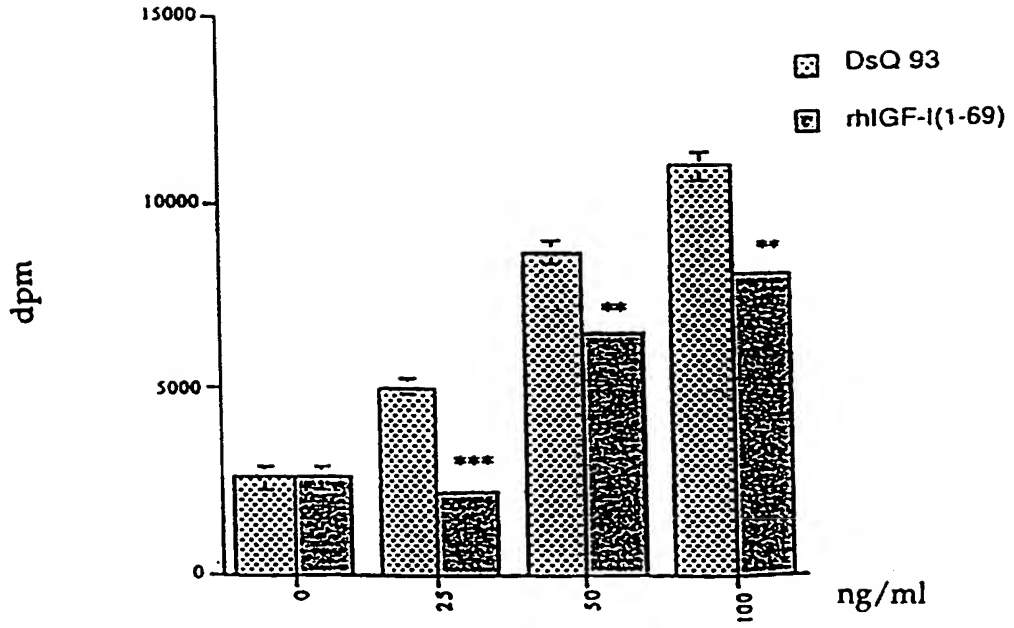
Lipogenesis in adipocytes



***p<0.001 vs. reference rhIGF-I, DsQ 93

Fig. 3

Lipogenesis in adipocytes



**p<0.01, ***p<0.001 vs. reference rhIGF-I, DsQ 93.

Fig. 4

Binding to IGF binding protein - 1

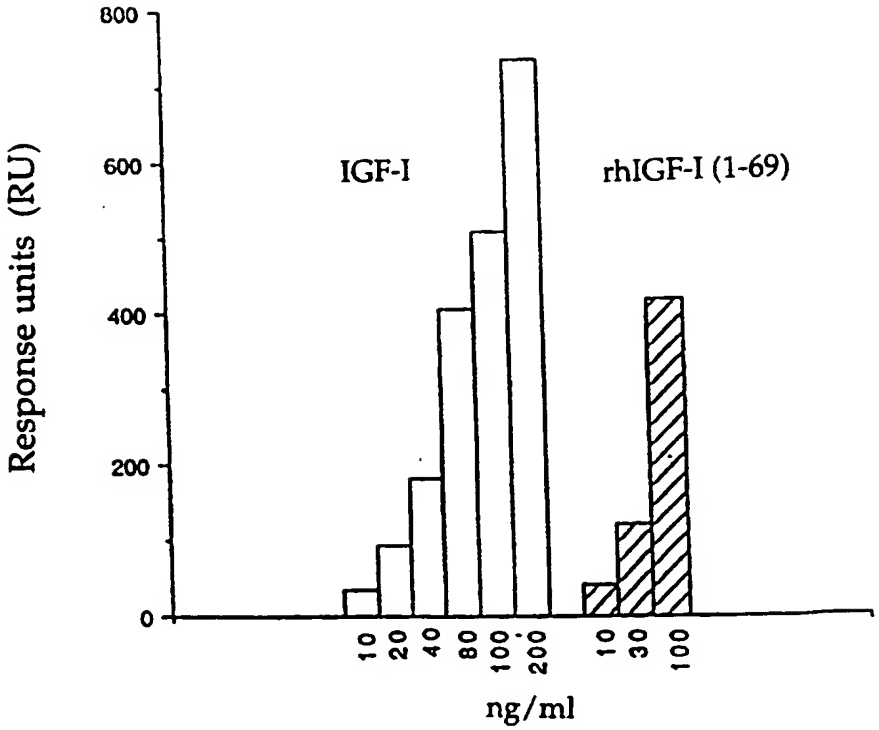


Fig. 5

Binding to IGF binding protein - 3

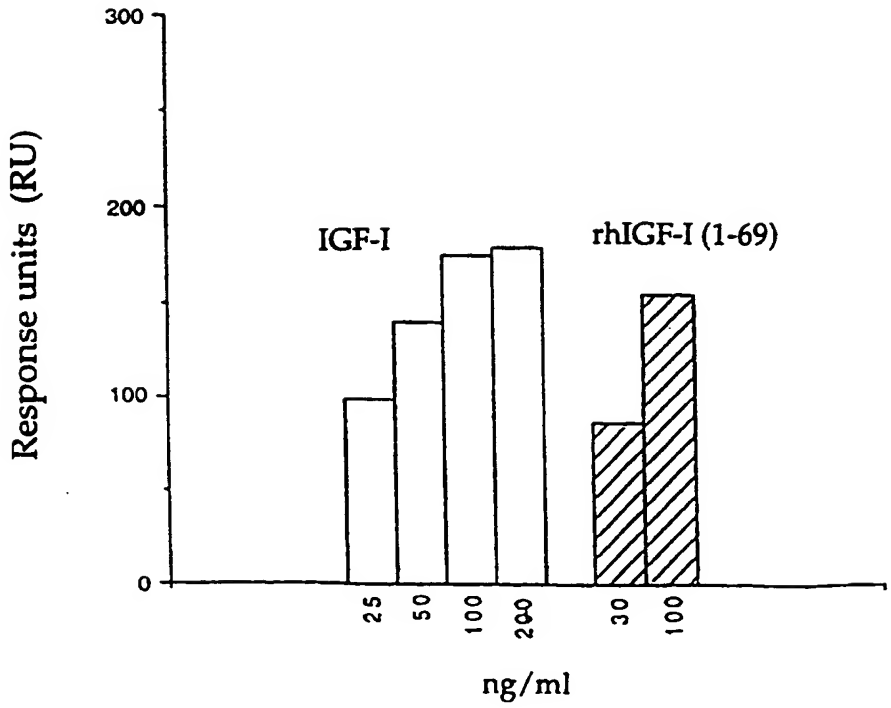


Fig. 6

Binding to the insulin receptor

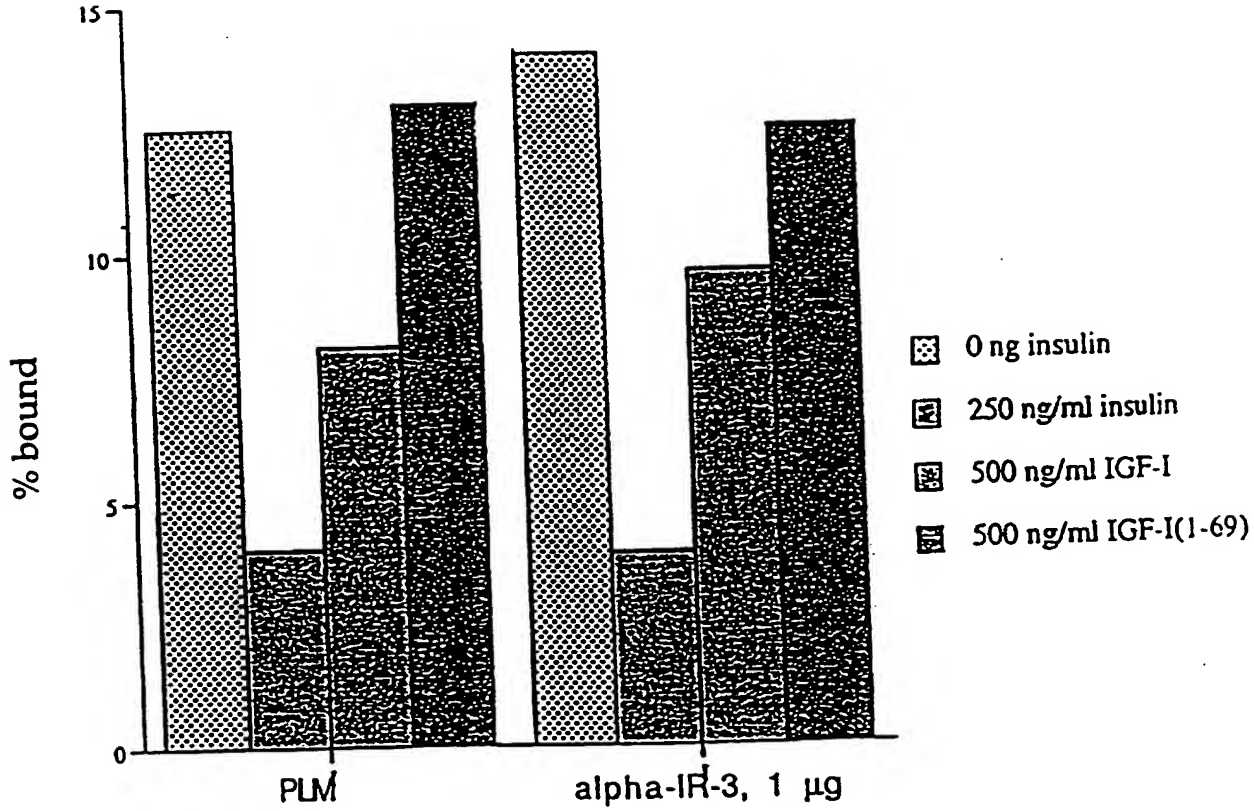


Fig. 7

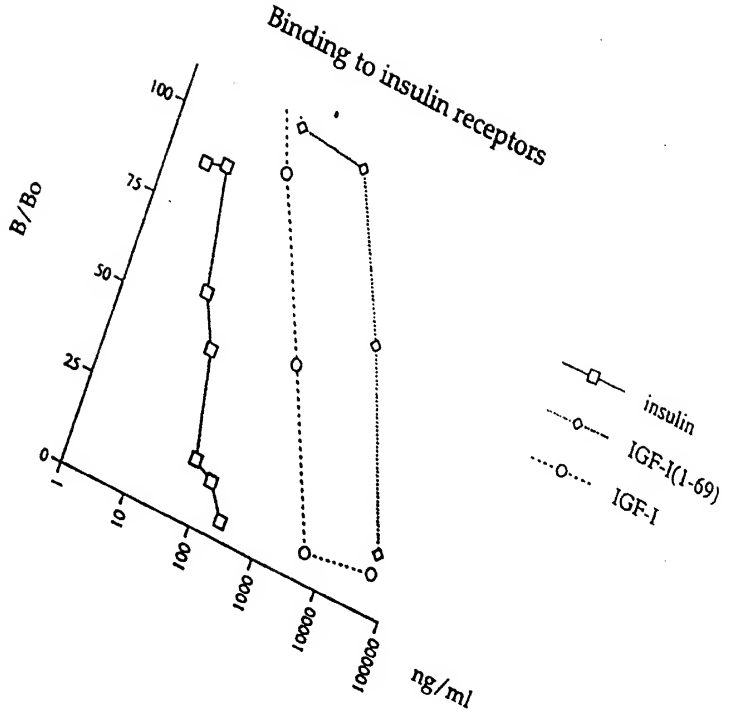

Binding to insulin receptors
B/Bo
100
75
50
25
0
1
10
100
1000
10000
100000
ng/ml
insulin
IGF-I(1-69)
IGF-I

Fig. 8 a

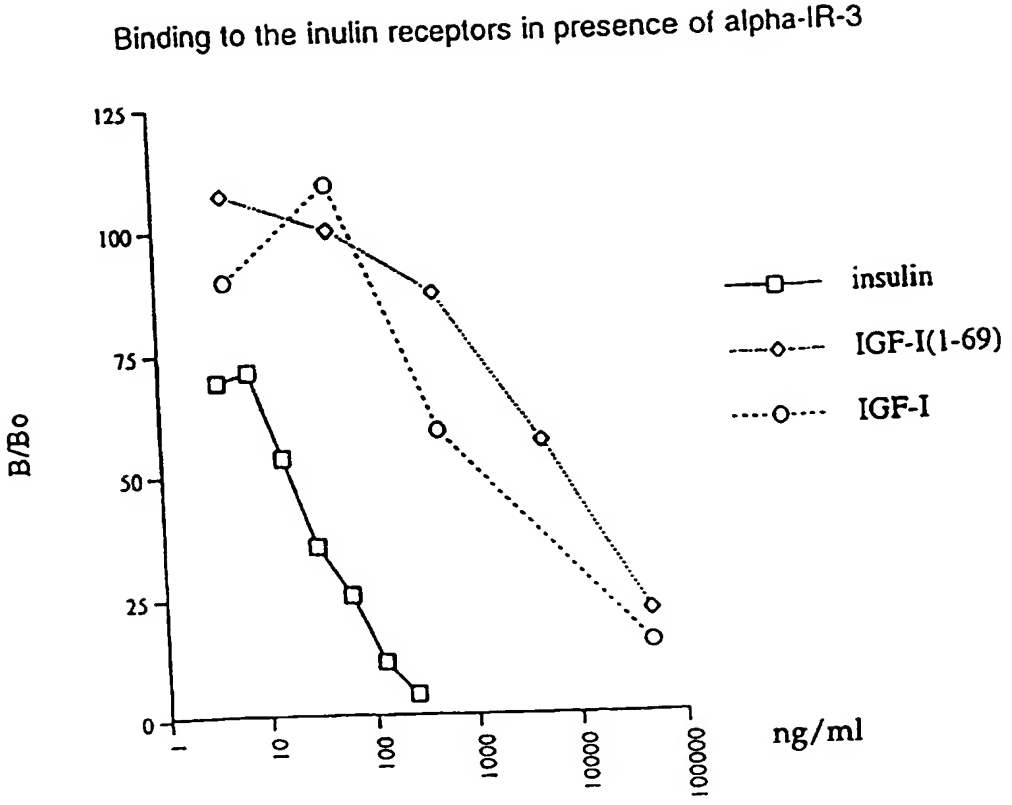

Binding to the inulin receptors in presence of alpha-IR-3
B/Bo
125
100
75
50
25
0
1
10
100
1000
10000
100000
ng/ml
insulin
IGF-I(1-69)
IGF-I

Fig. 8 b

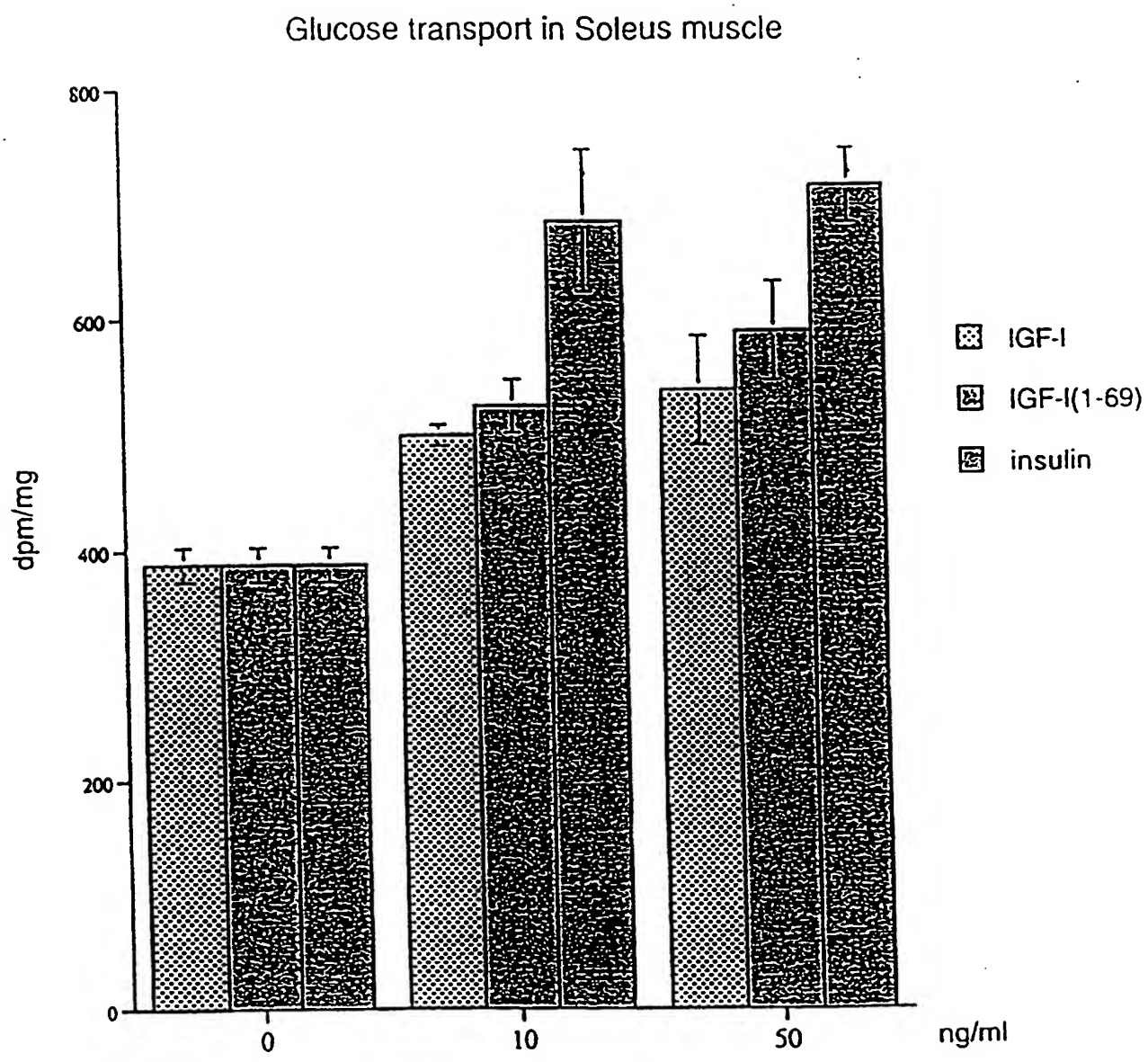

Glucose transport in Soleus muscle
800
600
400
200
0
dpm/mg
0
10
50
ng/ml
IGF-I
IGF-I(1-69)
insulin

Fig. 9

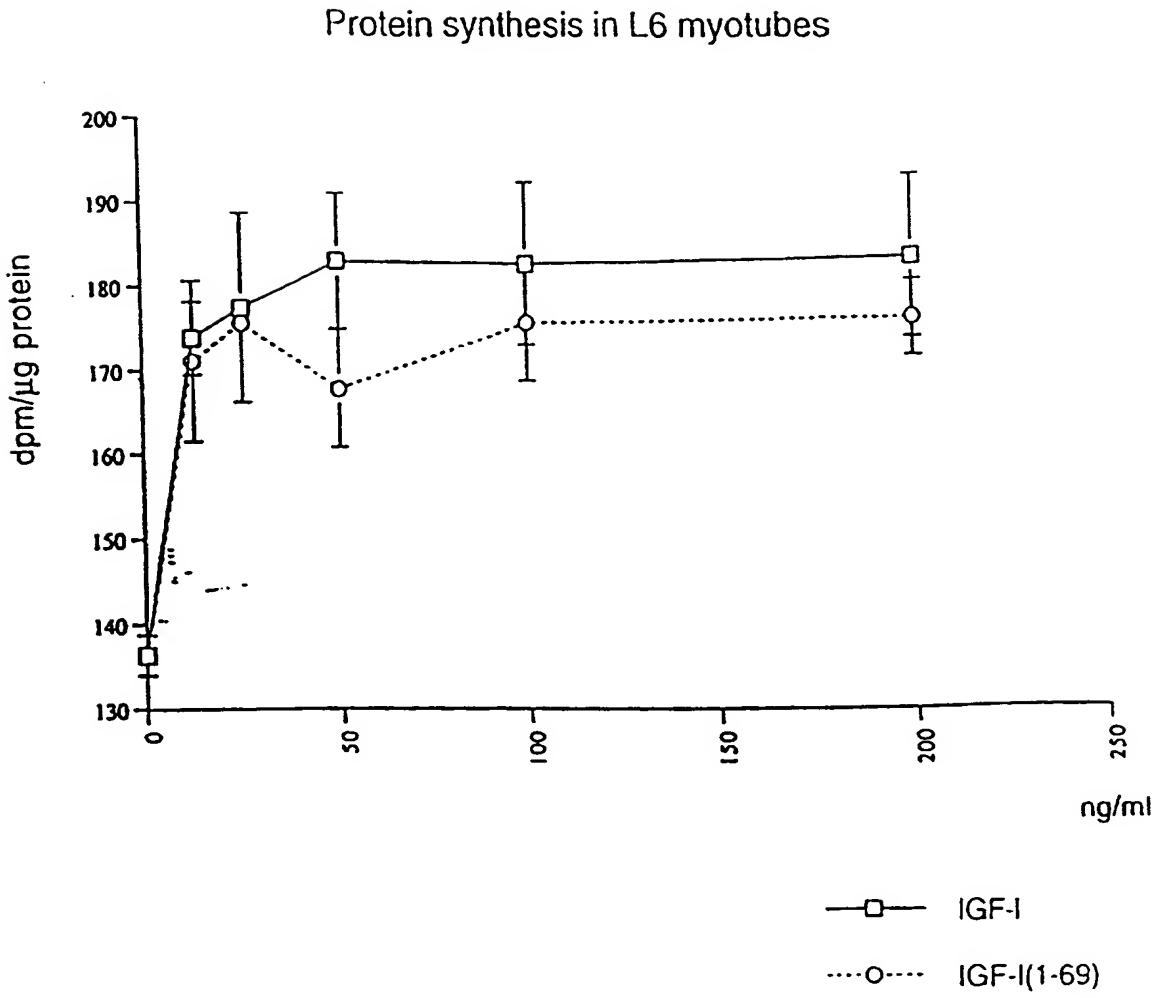

Protein synthesis in L6 myotubes
dpm/µg protein
200
190
180
170
160
150
140
130
0
50
100
150
200
250
ng/ml
IGF-I
IGF-I(1-69)

Fig. 10 a

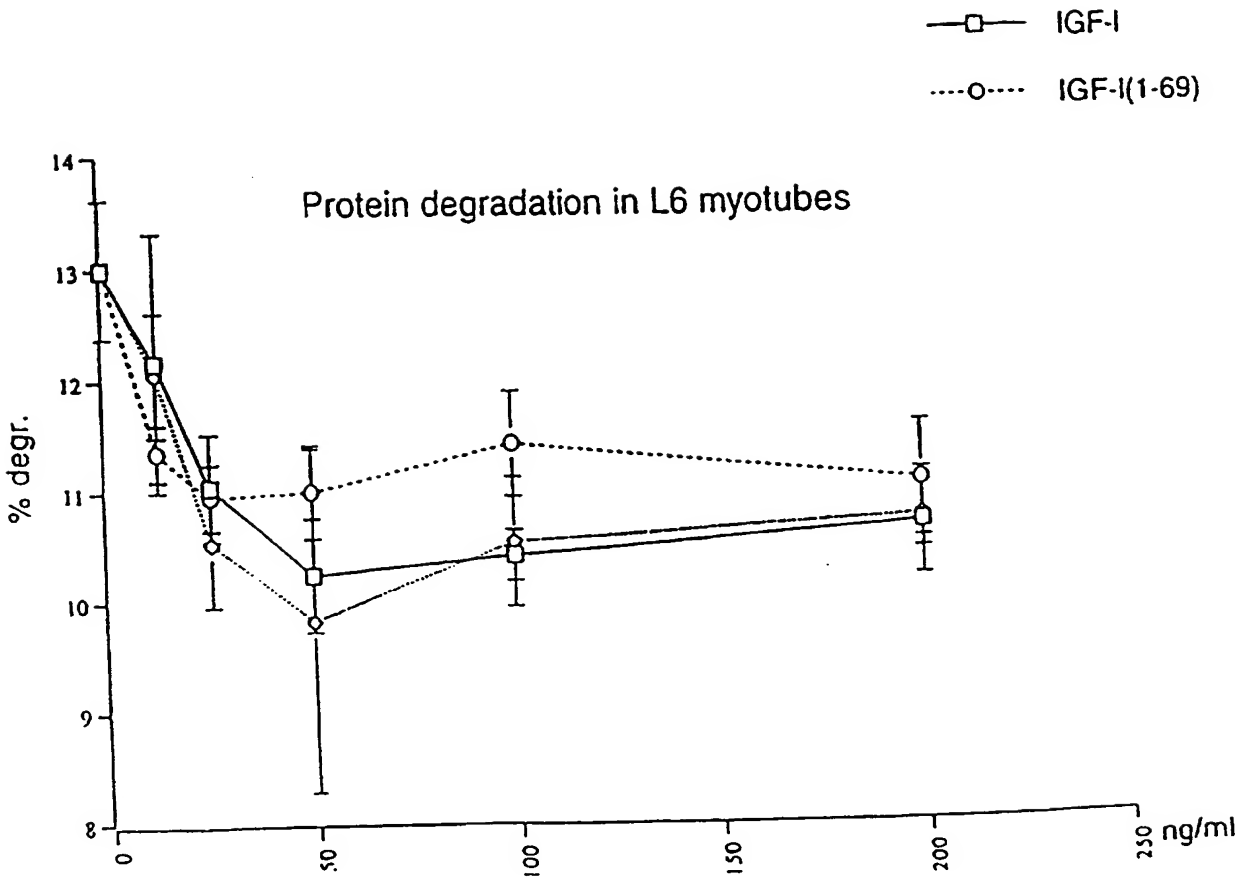

IGF-I
IGF-I(1-69)
Protein degradation in L6 myotubes
% degr.
14
13
12
11
10
9
8
0
50
100
150
200
250
ng/ml

Fig. 10 b

Tibia growth in vivo after 10 days of treatment

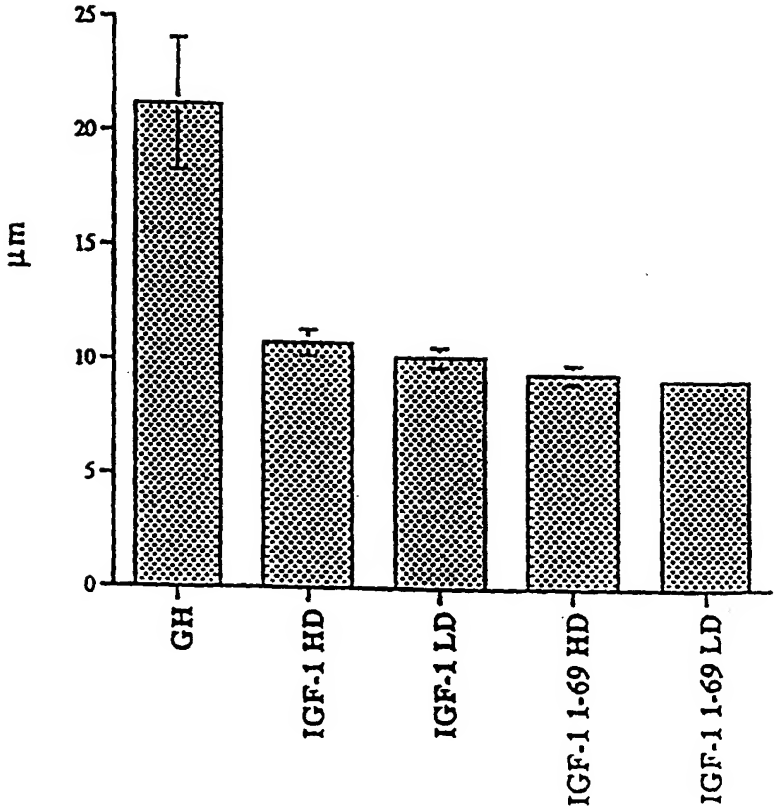


Fig. 11

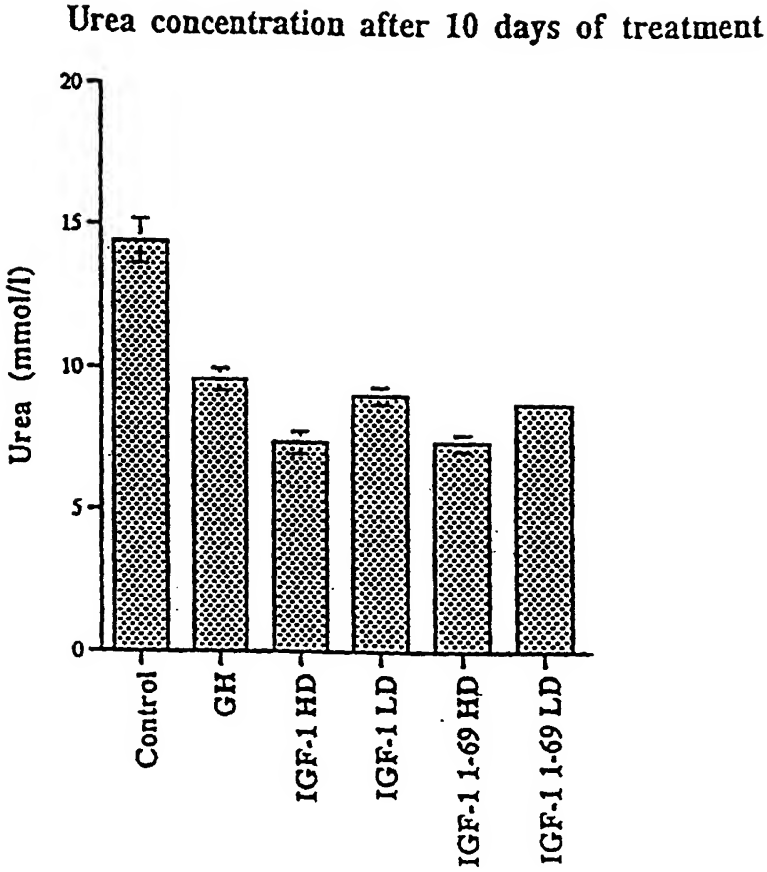

Urea concentration after 10 days of treatment
Urea (mmol/l)
20
15
10
5
0
Control
GH
IGF-1 HD
IGF-1 LD
IGF-1 1-69 HD
IGF-1 1-69 LD

Fig. 12

Glucose concentrations in hypox rat

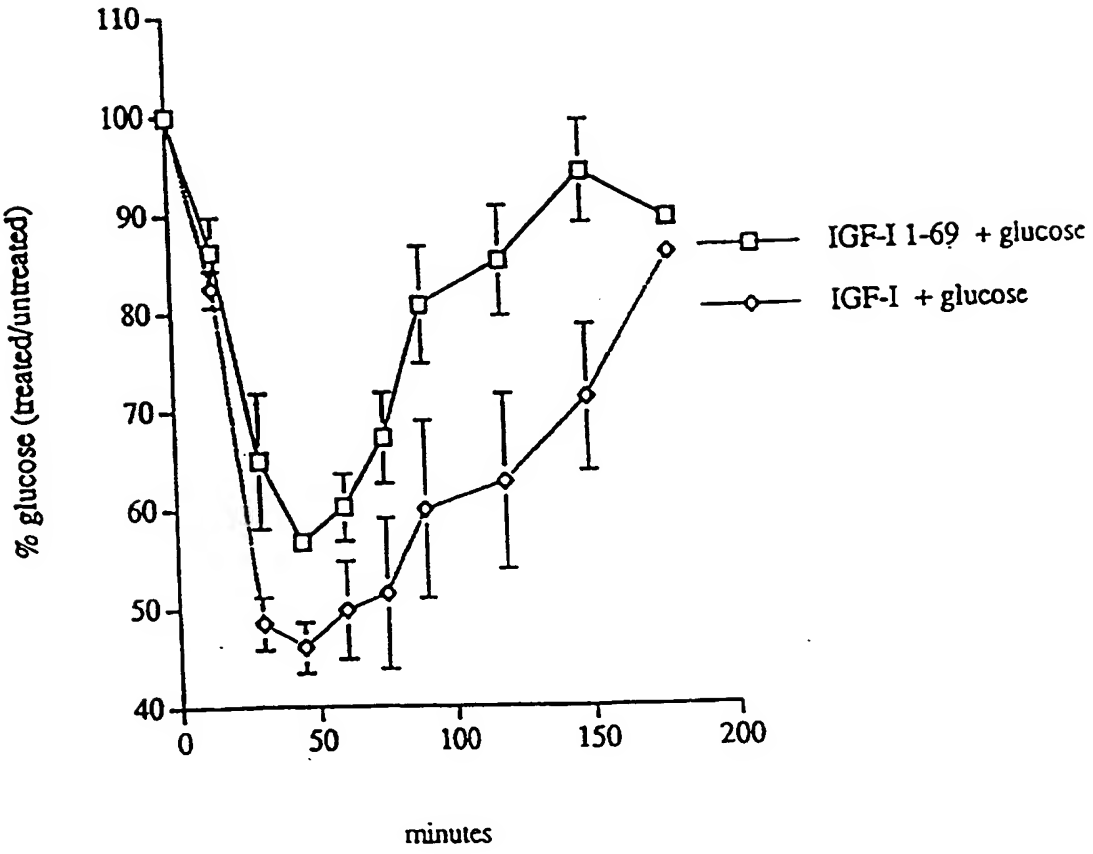


Fig. 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 96/00520

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/65, A61K 38/30
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

REG, CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9601275 A1 (PHARMACIA AB), 18 January 1996 (18.01.96), see claim 6 --	1-10
A	STN International, Derwent Information Ltd, WPIDS accession no. 89-118308, Sumitomo Seiyaku KK: "New insulin-like growth factor-1 derivs. - obtd. by condensn. of aminoacid units", JP,A,01063597, JP 87-221607 (870903) --	1-10
A	WO 9404569 A1 (THE AGRICULTURAL AND FOOD RESEARCH COUNCIL), 3 March 1994 (03.03.94) --	1-10

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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"E" ~~erlier document but published on or after the international~~ filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 July 1996	24 -07- 1996
Name and mailing address of the ISA/ Swedish Pat nt Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Gómez Lagerl¨f Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 96/00520

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9118621 A1 (GENENTECH, INC.), 12 December 1991 (12.12.91) --	1-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

01/07/96

International application No.
PCT/SE 96/00520

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9601275	18/01/96	NONE	
WO-A1- 9404569	03/03/94	NONE	
WO-A1- 9118621	12/12/91	EP-A,A- 0536226	14/04/93
		US-A- 5126324	30/06/92
		US-A- 5374620	20/12/94